JOURNAL OF BACTERIOLOGY, Aug. 1972, p. 437-442

Vol. 111, No. 2
Printed in U.S.A.

Spore Pool Glutamic Acid as a Metabolite in Germination

HAROLD F. FOERSTER

Biology Department, Sam Houston State University, Huntsville, Texas 77341

Received for publication 24 April 1972

Spore glutamic acid pools were examined in dormant and germinating spores using colorimetric and ^{14}C analytical procedures. Germination of spores of *Bacillus megaterium* (parent strain), initiated by D-glucose, was accompanied by a rapid drop in the level of spore pool glutamate, from 12.0 μg/mg of dry spores to 7.7 μg/mg of dry spores after 30 sec of germination. Similar decreases in extractable spore pool glutamate were observed with L-alanine-initiated germination of *B. licheniformis* spores. On the other hand, glutamate pools of mutant spores of *B. megaterium*, with a requirement of γ-aminobutyric acid for spore germination, remained unchanged for 9 min of germination, at which time more than 50% of the spore population had germinated. Evidence for conversion of spore pool glutamate to γ-aminobutyric acid during germination of spores of *B. megaterium* (parent strain) was obtained.

Spore formation in the genus *Bacillus* is accompanied by the accumulation of glutamic acid. Spores of three strains representing three species have been shown to contain large glutamic acid pools. In the strains examined, only one or a few other amino acids were accumulated, and at much reduced concentrations compared with glutamic acid. Glutamic acid accumulation in *B. megaterium* spores is independent of an exogenous supply of this amino acid, since the amount accumulated by spores grown in a sucrose-salts medium was not appreciably reduced compared with spores grown in a synthetic medium supplemented with glutamic acid or in supplemented nutrient broth (12).

Spore glutamate is tightly held, and is neither available for exchange with exogenous glutamic acid, nor is released in appreciable amounts during heat activation of spores. However, spore glutamate is released as free amino acid when spores are mechanically ruptured or when heated as aqueous suspensions at temperatures exceeding 90 C (12), suggesting that it is not covalently bonded to spore substance, and is, therefore, potentially available as a metabolite. The possible involvement of glutamic acid as a metabolite in spore germination received special significance with the isolation of mutant strains of *B. megaterium* with a requirement of γ-aminobutyric acid for spore germination (3). The requirement of γ-aminobutyric acid by these mutant strains could mean that decarboxylation of endogenous glutamic acid may constitute a critical metabolic event in physiological spore germination in the parent strain, but is blocked in the mutant strains. Experiments described in this paper support and enhance this view.

MATERIALS AND METHODS

Bacteria. The strains of *B. magaterium* used were the same strains described in a previous paper (3). *B. licheniformis* 10716 was obtained from the American Type Culture Collection, Rockville, Md.

Preparation of spores. The medium, growth conditions, and the methods used in the preparation and storage of spore suspensions have been described (3). Techniques used in the preparation of ^{14}C-labeled spores are described below. For germination testing, spores were heated as suspensions in demineralized water (9×10^8 viable spores per ml) for 60 min at 60 C. Samples of spore suspensions were freshly heated for each experiment; heated spores not used were discarded.

Optical density. Optical density of germinating spore suspensions was measured with a Spectronic 20 colorimeter at 540 nm.

Spores labeled with ^{14}C-L-glutamic acid. Labeled spores were produced in petri dishes on spore medium containing 0.1 g of L-glutamic acid per liter instead of the 1.0 g/liter usually employed. A 10-fold reduction in exogenous glutamate concentration permitted a more effective labeling of the spore pool glutamic acid. Consistent with previous findings (12),

no detectable changes in the amount of glutamate accumulated by spores grown in the presence of the two exogenous glutamate concentrations were observed. Each petri dish was inoculated with approximately 2×10^7 heated viable spores, which were spread evenly over the agar surface as a water suspension. The inoculated petri dishes were incubated at 37 C. After 8 hr of incubation, 2 μCi of L-glutamic acid-*U*-^{14}C in 0.3 ml of sterile water was added to each plate culture and uniformly mixed with the culture using a sterile bent glass rod. (Microscopic examination of 8-hr plate cultures with a phase-contrast microscope showed that 10 to 20% of the cells contained nonrefractile spores. However, less than 1% of the cells contained refractile spores.) Incubation at 37 C was continued until maximum free spores were obtained (24 to 36 hr). Spores were collected, washed, and stored as described (3).

Column chromatography. Individual amino acids were separated from spore extracts and recovered for quantitative analysis by preparatory column chromatography employing HCl gradient elution techniques (7). Dowex 50 $\times$ 8 resin (200 to 400 mesh) in the Na^+ form, previously washed and equilibrated with 1 N HCl, was poured as a slurry into a chromatographic tube (0.9 by 45 cm) with a 500-ml solvent reservoir. After settling, each resin column (40 cm) was washed with 30 ml of 1 N HCl. The sample was applied to the column with a capillary pipette and was washed into the column with 0.2 ml of 1 N HCl. A 100-ml amount of 2 N HCl was slowly poured into the reservoir. The column was operated at room temperature with a flow rate of 3.0 ml/hr; samples were collected at 30-min intervals. A resin column was used only once, thus each of the spore extracts was chromatographed on a freshly poured column. For the conversion of H^+ resin into the Na^+ form and the regeneration of used resin, we used the procedures described by Moore and Stein (11).

Total pool glutamic acid. Samples containing 25 mg (dry weight) of heated spores each were germinated in K_i (40 mM) and D-glucose (1 mM). To minimize lag and to insure a more rapid and synchronous germination, heated spore suspensions and germinant solutions were temperature-equilibrated separately at 40 C. Each sample was germinated in a 250-ml Erlenmeyer flask containing 120 ml of germinant solution and a Teflon-coated stirring bar (5 by 22 mm). Germination was at 40 C with continuous magnetic stirring. To halt metabolic activity associated with germination, each sample was transferred, at the designated time, to a 1-liter flask containing 100 ml of boiling demineralized water. Samples were maintained at 100 C for 30 min and autoclaved for 15 min to extract pool amino acids (12). The samples were centrifuged (10 min, 20,000 $\times$ *g*) and the volatile liquids of the supernatant fractions were removed in a rotary evaporator. The nonvolatile residue of each sample was dissolved in a minimum volume (1 to 2 ml) of demineralized water and stored frozen in a 1-dram specimen vial. Samples of dormant spores were prepared in the same manner, without germination.

Spore pool glutamic acid was separated from each of the crude extracts by column chromatography. Each sample was individually thawed and applied to a freshly prepared resin column. Column fractions, 1.5 ml each, were transferred to 50-ml beakers and evaporated to dryness. The dry residue of each fraction was dissolved in 0.5 ml of demineralized water, 5 μliters was spotted on Whatman no. 1 chromatography paper (different fractions were spotted 15 mm apart), and the chromatograms were developed descendingly with *n*-butanol–glacial acetic acid–water (65:15:25, v/v). L-Glutamic acid was spotted on each chromatogram for reference. Each developed chromatogram was dried and sprayed with Ninspray (Nutritional Biochemicals Corp., Cleveland, Ohio); drying and color development were at room temperature. Sample fractions containing glutamic acid, as shown by paper chromatograms, were pooled and evaporated to dryness, and the dry residue of each sample was dissolved in 1 ml of demineralized water. The samples were analyzed for glutamic acid using the colorimetric procedure of Rosen (15) and referred to a reference standard prepared with L-glutamic acid reagent obtained from J. T. Baker Chemical Co., Phillipsburg, N.J.

Residual pool ^{14}C-glutamic acid. Samples containing 2.5 mg of spores each (dry weight), grown in the presence of L-glutamic acid-*U*-^{14}C, were used. Heated spores, suspended in demineralized water and germinant solution (10 ml) and dispensed in 25 by 95-mm specimen vials (each with a 3 by 10-mm Teflon-coated stirring bar), were temperature-equilibrated separately at 40 C. Spores were germinated (KI, 40 mM; D-glucose, 1 mM) at 40 C with continuous stirring. Germinative activity was stopped by transferring each sample to a 125-ml Erlenmeyer flask containing 20 ml of boiling, demineralized water. The samples were extracted and prepared for analysis as described in the preceding section, with the following changes. (i) The dry residue of each sample was dissolved in 1 ml of demineralized water containing 2 mg of unlabeled carrier L-glutamic acid and stored in a frozen state pending column chromatography; (ii) column fractions containing glutamic acid (determined by paper chromatography) were pooled in a 50-ml beaker and dried. The dry residue of each pooled sample was dissolved in 50 μliters of demineralized water, transferred with a pipette (50 μliters) to a 2-cm paper disc (Whatman no. 3) which was impaled on two 1.5-inch specimen pins mounted on a styrofoam base, and was dried under a 250-w infrared lamp. The bottom of each sample beaker was washed with 2 volumes (50 μliters) of demineralized water, the latter transferred to the respective disc and dried. The dry sample discs were analyzed by liquid scintillation spectrometry.

^{14}C counting. Samples containing ^{14}C were analyzed in standard vials in a toluene-base liquid scintillation cocktail [2,5-diphenyloxazole and 1,4-bis-2-(5-phenyloxazoly)benzene]. Counting was done in a Beckman LS 200 scintillation spectrophotometer optimized for ^{14}C.

RESULTS

To assess the involvement of glutamic acid in physiologically germinating spores of *B. megaterium* QM B1551 (parent strain), glutamic acid pools, extracted from dormant and germinating spores and separated from other amino acids by preparatory column chromatography, were compared. Fig. 1 depicts two parameters of spore glutamic acid pools in the parent strain: (i) total pool glutamic acid (closed circles) determined colorimetrically, and (ii) residual pool glutamic acid (closed squares) based on ^{14}C activities of pools labeled with glutamic acid-*U*-^{14}C.

Heat-activated spores of *B. megaterium* QM B1551 were found to contain 12.0 μg of heat-extractable glutamic acid per mg of dry spores (based on three separate determinations of 12.2, 11.1, and 12.5 μg/mg of dry spores). Germination of heated spores in solutions of KI (40 mM) and D-glucose (1 mM) was accompanied by rapid changes in heat-extractable glutamic acid. Thirty seconds after the initiation of germination, the total pool glutamic acid dropped from an initial level of 12.0 μg to 7.7 μg per mg of dry spores. After the initial drop, however, the amount of pool glutamic acid rose sharply in germinating spores, reaching a level of 15.0 μg/mg of dry spores at the end of 3 min of germination, and leveling off at approximately 16.0 μg/mg of dry spores after 6 and 9 min of germination (the latter two intervals were not included in Fig. 1). A metabolically labile spore glutamic acid pool was indicated. The drop in extractable glutamic acid during the early moments of physiological spore germination was of particular interest; a possible involvement of glutamic acid metabolism was indicated. The sharp increase in extractable glutamic acid after 30 sec of germination indicated that the germinating spores quickly acquired the means for generating glutamic acid. The source of the newly formed glutamic acid, whether the result of a de novo synthesis or the breakdown of glutamic acid-containing spore components (14), was not investigated.

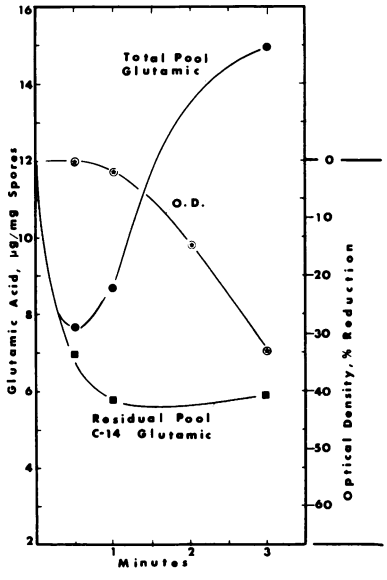

FIG. 1. *Total pool glutamic acid and residual pool ^{14}C-glutamic acid in dormant and germinating spores of B. megaterium. Total pool glutamic acid was measured colorimetrically. Glutamic acid-U-^{14}C extracted from spores was measured using standard scintillation spectrometry. Sample counts, corrected for background, ranged from 2.4 × 10^3 counts/min in dormant spores to 1.16 × 10^3 counts/min after 60 sec of germination. The count obtained with dormant spores was set equal to 12.0 μg of glutamic acid per mg of dry spores (a value established colorimetrically). Residual pools obtained with germinating spores were computed from the dormant spore pool. Heated spores were germinated in solutions containing KI (40 mM) and D-glucose (1 mM) at 40 C.*

Proof of the metabilism of pool glutamic acid is paramount to its implication as a metabolite in spore germination. The examination of total extractable glutamic acid pools from dormant and germinating spores indicated that pool glutamic acid was metabolized by germinating spores of the parent strain. Additional evidence was obtained by comparing glutamic acid pools labeled with glutamic acid-*U*-^{14}C. These results are shown in Fig. 1 as residual pool ^{14}C-glutamic acid. The loss of radioactivity from the labeled glutamic acid pools of germinating spores during the early moments of germination coincided with the loss of glutamic acid measured colorimetrically. As expected, the radioactivity curve (closed squares) did not reverse itself at 30 sec of germination, but continued to drop, reaching a low point at 60 sec. A slight increase in ^{14}C activity was observed at 3 min of germination, possibly the result of ^{14}C label added to the glutamic acid pool from nonspecifically labeled spore components.

These results indicate that glutamic acid pools were rapidly metabolized by parent spores during physiological germination. Comparison of the kinetics in the loss of glutamic acid from spore pools and the reduction in optical density of germinating spores was of interest. Loss in optical density of germinating

spore suspensions consistently showed a lag of approximately 1 min, whereas no lag was apparent in the loss of spore glutamic acid. Thus, physical changes, such as loss of refractility, which accompany spore germination may result from, or be dependent on, specific metabolic events in physiologically germinating spores.

Residual pool ^{14}C-glutamic acid in germinating spores of strain γ065. Strong presumptive evidence in support of endogenous glutamic acid as a potentially important metabolite in spore germination derives a priori from the fact that mutant spore strains, having a specific requirement for exogenous γ-aminobutyric acid, can be obtained (3). A requirement of γ-aminobutyric acid by these strains for spore germination may result from their inability to metabolize (decarboxylate) endogenous glutamic acid. This possibility was examined by using spores of strain γ065 labeled with glutamic acid-*U*-^{14}*C*. Spores of strain γ065 contain glutamic acid pools equal in size to the pool size in the parent strains, i.e., 12 μg/mg of dry spores.

Unlike the parent strain, ^{14}C counts, obtained as extractable glutamic acid-*U*-^{14}*C* from dormant and germinating spores of strain γ065, remained unchanged during the first 9 min of germination (Fig. 2). At the end of 9 min, more than half of the spores had germinated, as indicated by a 38% drop in optical density. The stable nature of the glutamic acid pools in germinating mutant spores suggests that the decarboxylation of endogenous glutamic acid may be required for physiological spore germination by the parent strain. The rapid germination obtained with the γ-aminobutyric acid-dependent mutant spore suspensions suggests that this compound relieves this requirement (see also 3).

Residual pool ^{14}C-glutamic acid in germinating spores of *B. licheniformis* 10716. The requirements for the initiation of physiological spore germination differ for different species. Indeed, strains of any one species may show considerable variation in their germination requirements (4).

A point of interest is whether spore glutamic acid pools are similarly affected in germinating spores of strains which have different germination requirements. Heated spore suspensions of *B. licheniformis* 10716 can be rapidly and completely germinated by 1 mM of L-alanine in solutions of a suitable salt. Dormant spores of this strain contained 9.7 μg of heat-extractable glutamic acid per mg of dry spores.

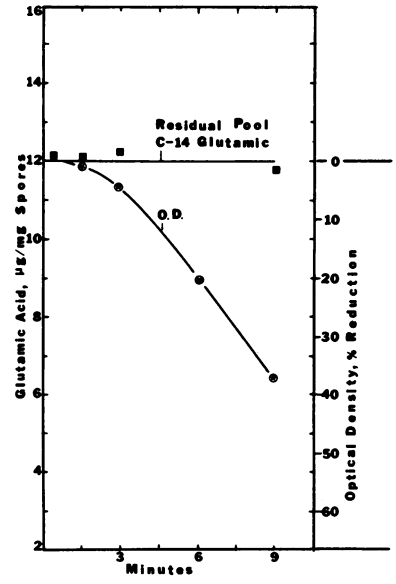

FIG. 2. *Residual pool ^{14}C-glutamic acid in dormant and germinating spores of mutant strain γ065. Glutamic acid-U-^{14}C extracted from spores was assayed. Sample counts, corrected for background, ranged from 2.15 × 10^3 counts/min to 2.3 × 10^3 counts/min. The count obtained with dormant spores was set equal to 12.0 μg of glutamic acid per mg of dry spores. Residual pools obtained with germinating spores were computed from the dormant spore pool. Heated spores were germinated in KI (40 mM) and L-alanine and γ-aminobutyric acid (1 mM each) at 40 C.*

L-Alanine-induced germination is accompanied by a rapid drop in the residual pool of ^{14}C-glutamic acid in spores of *B. licheniformis* labeled with glutamic acid-*U*-^{14}*C* (Fig. 3). The results obtained with *B. licheniformis* showed a marked resemblance to the results obtained with the parent strain of *B. megaterium* (Fig. 1). Only a slight difference in the rate of loss of ^{14}C label from the glutamic acid pools was observed. Approximately 52% of the initial label was lost from the *B. megaterium* pools at the end of 60 sec, a similar loss of label from the *B. licheniformis* pools was observed after approximately 2.5 min of germination, and a loss of 56% was obtained after 4 to 5 min of germination.

Transfer of ^{14}C to exogenous γ-aminobutyric acid by germinating spores labeled with glutamic acid-U-^{14}C. Evidence supporting the conversion of spore pool glutamic acid to γ-aminobutyric acid in *B. megaterium*, parent strain, during D-glucose-initiated spore germination was obtained (Fig. 4). Heated

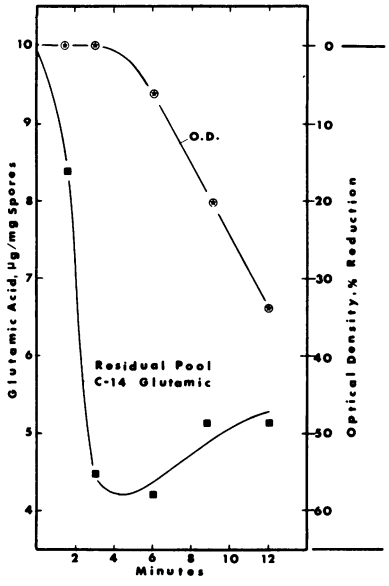

FIG. 3. *Residual pool ^{14}C-glutamic acid in dormant and germinating spores of B. licheniformis 10716. Glutamic acid-U-^{14}C extracted from spores was assayed. Sample counts, corrected, ranged from 1.1 × 10^4 counts/min in dormant spores to 4.6 × 10^3 counts/min after 6 min of germination. The count obtained with dormant spores was set equal to 9.7 µg of glutamic acid per mg of dry spores. Residual pools obtained with germinating spores were computed from the dormant spore pool. Heated spores were germinated in solutions containing K-acetate (40 mM) and L-alanine (1 mM) at 40 C.*

spores labeled with glutamic acid-*U*-^{14}C were germinated in a medium containing KI (40 mM), D-glucose (1 mM), and unlabeled γ-aminobutyric acid (1 mM). Germination and extraction procedures were identical to those described. Samples of dormant spores, and samples of spores germinated for periods of 30, 90, and 180 sec were compared. The glutamic acid and the γ-aminobutyric acid in each sample were separated by preparatory column chromatography and assayed for radioactivity.

Substantial increases in ^{14}C activity were recovered in the γ-aminobutyric acid fractions coincident with the decreases in glutamic acid-*U*-^{14}C. After 3 min of germination, assuming that all or most of the ^{14}C label in the γ-aminobutyric acid was directly derived from spore pool glutamic acid, approximately 17% of the glutamic acid was decarboxylated and recovered in the γ-aminobutyric acid fractions. Since 20% of the label was lost as $^{14}CO_2$, the recovery was in fact somewhat higher.

DISCUSSION

The isolation of mutant strains of *B. megaterium* QM B1551, with a specific requirement of exogenously added γ-aminobutyric acid for spore germination, emphasizes the possible involvement of endogenous glutamic acid metabolism in spore germination (3). This receives additional appeal when one considers that the dormant spores of at least four *Bacillus* species contain relatively large glutamic acid pools (12). That the initiation of spore germination in heated spores of *B. megaterium* may be dependent upon the endogenous metabolism of glutamic acid is supported by results reported in this paper.

Germination of heated parent spore suspensions, by solutions of D-glucose (1 mM) and KI (40 mM), was accompanied by a rapid decrease in extractable glutamic acid levels. Independent analyses of total glutamic acid pools and residual glutamic acid-*U*-^{14}C pools showed similar decreases in heat-extractable glutamic acid during the early moments (30 sec or less) of spore germination (fig. 1). Subsequent to the initial drop, however, a rapid recovery in extractable pool glutamic acid was observed in the total pool analyses. The rapid increase in pool glutamic acid levels probably represents one phase of a complex and vigorous metabolic

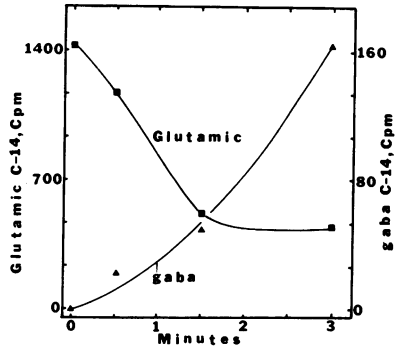

FIG. 4. *Transfer of ^{14}C to exogenous γ-aminobutyric acid by germinating spores labeled with L-glutamic acid-U-^{14}C. Heated spores labeled with L-glutamic acid-U-^{14}C were germinated in a medium containing KI (40 mM), D-glucose (1 mM) and unlabeled γ-aminobutyric acid (1 mM). Samples obtained after 30, 90, and 180 sec of germination were extracted, and the glutamic acid and γ-aminobutyric acid (gaba) in each sample were separated by preparatory column chromatography and assayed for ^{14}C activity. Equal samples of dormant spores, prepared and analyzed in the same manner, were included for comparison. All points plotted were corrected for background.*

activity associated with spore germination (6, 8–10, 13, 16, 17).

A second point of interest, and potentially important in spore germination, was the finding that glutamic acid pools in mutant spores (γ065) requiring exogenous γ-aminobutyric acid remained unchanged during the first 9 min of germination (Fig. 2). This contrasted with the glutamic acid pool levels in the spores of the parent strain which were reduced to approximately 50% of the initial levels after 60 sec of germination. Similar losses, although 3 to 4 min of germination were required, were observed with L-alanine-initiated germination in *B. licheniformis* (Fig. 3). Viewed in this context, the rapid metabolism of spore pool glutamic acid during physiological germination argues well for the presence of glutamic acid decarboxylase in bacterial spores. The presence of glutamic acid decarboxylase in spores was also indicated by the rapid transfer of ^{14}C from spore pool L-glutamic acid-*U*-^{14}C to exogenously added unlabeled γ-aminobutyric acid during spore germination (Fig. 4).

Several amino acid decarboxylases have been described in vegetative bacteria (5). L-Glutamic acid decarboxylase has been demonstrated in gram-negative and gram-positive bacteria, including vegetative cells of *Bacillus* (1, 2, 18). Studies on L-glutamic acid decarboxylase activities in spores of *B. megaterium* are in progress, and the results of these studies will be reported separately.

ACKNOWLEDGMENTS

I thank Manley Mandel for his generous help. The assistance of Frances Blount is gratefully acknowledged.

LITERATURE CITED

1. Akers, E., and J. N. Aronson. 1971. Detection on polyacrylamide gels of L-glutamic acid decarboxylase activities from *Bacillus thuringiensis*. Anal. Biochem. **39**: 535–538.
2. Cozzani, I., A. Misuri, and C. Santoni. 1970. Purification and general properties of glutamate decarboxylase from *Clostridium perfringens*. Biochem. J. **118**: 135–141.
3. Foerster, H. F. 1971. γ-Aminobutyric acid as a required germinant for mutant spores of *Bacillus megaterium*. J. Bacteriol. **108**:817–823.
4. Foerster, H. F., and J. W. Foster. 1966. Response of *Bacillus* spores to combinations of germinative compounds. J. Bacteriol. **91**:1168–1177.
5. Gale, E. F. 1946. The bacterial amino acid decarboxylases, p. 1–32. *In* F. F. Nord (ed.), Advances in enzymology, vol. 6. Interscience Publ. Inc., N.Y.
6. Halvorson, H., and B. D. Church. 1957. Intermediate metabolism of aerobic spores. II. The relationship between oxidative metabolism and germination. J. Appl. Bacteriol. **20**:359–372.
7. Hirs, C. H. W., S. Moore, and W. H. Stein. 1954. The chromatography of amino acids on ion exchange resins: use of volatile acids for elution. J. Amer. Chem. Soc. **76**:6063–6065.
8. Levinson, H. S., and M. T. Hyatt. 1955. The stimulation of germination and respiration of *Bacillus megaterium* spores by manganese, L-alanine, and heat. J. Bacteriol. **70**:368–374.
9. Levinson, H. S., and M. T. Hyatt. 1956. Correlation of respiratory activity with phases of spore germination and growth in *Bacillus megaterium* as influenced by manganese and L-alanine. J. Bacteriol. **72**:176–183.
10. Mandels, G. R., H. S. Levinson, and M. T. Hyatt. 1956. Analysis of respiration during germination and enlargement of spores of *Bacillus megaterium* and the fungus *Myrothecium verrucaria*. J. Gen. Physiol. **39**: 301–309.
11. Moore, S., and W. H. Stein. 1951. Chromatography of amino acids on sulfonated polystyrene resins. J. Biol. Chem. **192**:663–681.
12. Nelson, D. L., and A. Kornberg. 1970. Biochemical studies of bacterial sporulation and germination. XVIII. Free amino acids in spores. J. Biol. Chem. **245**: 1128–1136.
13. O'Conner, R., and H. Halvorson. 1959. Intermediate metabolism of aerobic spores. IV. Alanine deamination during the germination of spores of *Bacillus cereus*. J. Bacteriol. **78**:844–851.
14. Powell, J. F., and R. E. Strange. 1953. Biochemical changes occurring during the germination of bacterial spores. Biochem. J. **54**:205–209.
15. Rosen, H. 1957. A modified ninhydrin colorimetric analysis for amino acids. Arch. Biochem. Biophys. **67**:10–15.
16. Setlow, P., and A. Kornberg. 1970. Biochemical studies of bacterial sporulation and germination. XXII. Energy metabolism in early stages of germination of *Bacillus megaterium* spores. J. Biol. Chem. **245**:3637–3644.
17. Setlow, P., and A. Kornberg. 1970. Biochemical studies of bacterial sporulation and germination. XXIII. Nucleotide metabolism during spore germination. J. Biol. Chem. **245**:3645–3652.
18. Strausbauch, P. H., and E. H. Fischer. 1970. Chemical and physical properties of *Escherichia coli* glutamate decarboxylase. Biochemistry **9**:226–233.